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Osteogenic protein-1 promotes the formation of tissue-engineered cartilage using the alginate-recovered-chondrocyte method

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Summary

Objective: This study examined the effects of a growth factor, recombinant human osteogenic protein-1 (rhOP-1), on the formation of tissue-engineered cartilaginous tissue by adult bovine articular chondrocytes using the alginate-recovered-chondrocyte (ARC) method.

Design: To ascertain if rhOP-1 enhances the formation of the cell-associated matrix (CM) and the characteristics of CM formation, bovine articular chondrocytes were first cultured for up to 14 days in alginate beads in medium supplemented with serum, with or without rhOP-1. Then, the recovered chondrocytes and their associated CM were resuspended in medium, with or without OP-1, seeded onto culture inserts, and incubated for an additional 14 days. The fabricated ARC tissues were subjected to biochemical and histological analyses.

Results: The addition of rhOP-1 to the medium in the alginate bead culture step resulted in an increased accumulation of both proteoglycan (PG) and collagen, with a ratio of PG to collagen that was higher than that found in native adult cartilage. The addition of rhOP-1 in the second step had a similar stimulatory effect during 14 days of culture. Histological examination of the tissue formed under all conditions revealed a cartilage-like matrix, stained strongly by toluidine blue. The thickness of the tissues obtained from culture conditions that included the addition of rhOP-1 was four times greater than that of the tissues cultured without rhOP-1.

Conclusions: Using the ARC method, rhOP-1 enhanced the formation of matrix and generated a voluminous tissue-engineered cartilaginous construct. These characteristics may be beneficial in generating constructs that can cover large defects.

Key words: Cartilage, Alginate, Tissue culture, Tissue engineering, Bone morphogenetic protein-7, Osteogenic protein-1.

Introduction

Articular cartilage is a specialized avascular tissue composed of a small number of cells and an extensive extracellular matrix. In synovial joints, cartilage provides a resilient, load-bearing surface with low friction during movement. Once damaged, articular cartilage has limited intrinsic capacity for repair[1]. In adult animals, the response to full-thickness defects extending to the subchondral bone is the formation of a fibrocartilaginous repair tissue[2] that has a biochemical composition different from that of normal cartilage. The repair tissue contains relatively low amounts of aggrecan and type II collagen, markers of the chondrocytic phenotype[3], and relatively high amounts of type I collagen, a protein not present in measurable amounts in normal adult articular cartilage[4]. The abnormal composition of the repair tissue results in poor biomechanical function[5]. Due to the ineffective intrinsic repair of articular defects, methods for promoting cartilage repair are under active investigation[6].

An *in vitro* engineered cell-laden cartilaginous tissue may provide an alternative graft material that can easily be inserted into a cartilage defect to facilitate structural repair. Cartilaginous tissues have been synthesized using a variety of cell types. Immature chondrocytes or chondroprogenitor cells have often been used and are typically mixed with or infiltrated into a degradable synthetic scaffold material, such as polylactic acid and polyglycolic acid, or natural materials derived from or analogous to collagen, hyaluronan or fibrin[7]. Alternatively, methods have been developed to form implants that are composed only of cells

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and their products. The formation of a cartilaginous tissue has been described using immature chondrocytes in the absence of a scaffold in high-density monolayer cultures[8,9]. When cultured under the same conditions, chondrocytes from adult articular cartilage form only a small amount of matrix[8]. Recently, a novel scaffold-free technique, termed the alginate-recovered-chondrocyte (ARC) method, was introduced for the formation of cartilaginous tissue *in vitro* from bovine adult articular chondrocytes[10]. The ARC method consists of the culture of adult articular chondrocytes in alginate for a period of time sufficient to generate a cell-associated matrix (CM). Then, the cells with their CM are recovered and allowed to integrate into a cartilaginous tissue, with cell and matrix phenotypic properties typical of articular cartilage[10]. For practical applications, acceleration of the formation of cartilaginous tissue is desirable during *in vitro* culture. The inclusion of exogenous growth factors may stimulate cell proliferation and matrix deposition during the culture period[11–13].

Bone morphogenetic proteins (BMPs), also termed osteogenic proteins, are members of the transforming growth factor β (TGF-β) superfamily of proteins[14]. BMPs are growth and differentiation factors that induce mesenchymal precursor cells to differentiate into bone via endochondral ossification. Osteogenic protein-1 (OP-1), otherwise known as BMP-7, is an effective stimulator of the synthesis of matrix components, such as proteoglycans (PGs)[15] and collagen, by chondrocytic cells[16]. Histological analysis has suggested that OP-1 enhances the formation of CM by adult chondrocytes[17,18]. However, quantitative analysis is needed to clarify the extent of such an effect. In addition, the effects of OP-1 on the formation of cartilaginous tissue using the ARC method remain to be established.

The objectives of the current study were to determine: (1) the effects of OP-1 on the formation of CM in the alginate culture system and the production of a cartilaginous tissue when these cells and their CM are subsequently cultured on a porous insert; and (2) if the PG and collagen components that accumulate in the matrix of the cartilage constructs are representative of those present in normal articular cartilage.

Material and methods

All chemicals and reagents were of highest available analytical grades from Sigma (St. Louis, MO) unless otherwise stated.

CHONDROCYTE HARVEST AND CULTURE IN ALGINATE

Chondrocytes were harvested and cultured in alginate beads, essentially as previously described[19,20]. Briefly, articular cartilage was dissected from metacarpophalangeal joints of adult bovine animals (18–24 months old, four hooves per experiment) and chondrocytes were isolated. Freshly isolated cells were encapsulated in 1.2% low viscosity alginate solution (Keltone LV, a gift from ISP alginate, San Diego, CA) at 4 million cells/ml and the newly formed beads (containing approximately 40,000 cells/bead) were cultured in medium [50/50 Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Cellgro-Mediatech, Herndon, VA), supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 10 μg/ml gentamicin (Invitrogen Life Technologies, Carlsbad, CA), 360 μg/ml L-glutamine (Cellgro-Mediatech) and 25 μg/ml ascorbic acid].

EFFECTS OF OP-1 ON THE FORMATION OF CM IN ALGINATE BEADS

The effect of OP-1 on the accumulation of PGs and collagen in the CM in alginate culture was assessed using three different preparations of chondrocytes. Samples, each consisting of triplicate sets of nine beads, were placed in individual wells of a 24-well plate (Corning Costar Corp., Cambridge, MA) and cultured in 0.4 ml of complete medium alone or with OP-1 supplementation (100 ng/ml, a kind gift from Stryker Biotech, Hopkinton, MA; a concentration selected based on preliminary studies indicating this is the minimum dose that consistently induces a significant stimulation). The cultures were maintained for up to 14 days at 37°C in a humidified atmosphere of 5% CO_2, with daily changes of medium. On days 3, 7, 10 and 14, the beads were collected and the two matrix compartments (the CM and the further removed matrix [FRM]) were separated. Briefly, alginate beads containing encapsulated articular chondrocytes were dissolved by incubation in a chelating solution [55 mM sodium citrate, 0.15 M sodium chloride, pH 6.8]. The resulting suspension was centrifuged at 110 *g* for 10 min at 4°C to separate the cells with their CM in the pellet, from the constituents of the FRM in the supernatant[19,21]. The chondrocyte-CM samples were analyzed biochemically as described below. Cell viability in the CM fraction was quantified by manual counting on five different fields, using a fluorescent LIVE/DEAD® cell assay (Molecular Probes, Inc., Eugene, OR) in accordance with the manufacturer's suggested protocol.

The molecular constituents of the CM component were analyzed by histochemistry and immunohistochemistry using the well-characterized monoclonal antibody (1/20/5-D-4: MP Biomedicals, Irvine, CA) specific for a highly sulfated keratan sulfate (KS) epitope on aggrecan[19].

EFFECTS OF OP-1 ON THE FORMATION OF CARTILAGINOUS TISSUE USING THE ARC METHOD

The effect of OP-1 on the formation of cartilaginous tissue using the ARC method was examined. Chondrocytes were isolated and alginate beads were formed as described above. Seventy-five beads were incubated in triplicate in 12-well multiwell plates with 3 ml of complete medium without (control group) or with OP-1 (OP-1 group, 100 ng/ml). After 7 days of preculture in alginate beads, the medium was removed, the beads were dissolved, and the cells with their CM were obtained by mild centrifugation, as described above (Fig. 1). The resulting pellet containing "alginate-recovered cells" with their CM was then resuspended in complete medium (1 ml/75 beads) supplemented without (control group) or with OP-1 (OP-1 group, 100 ng/ml) (Fig. 1). Transwell™ cell culture inserts (0.4 μm pore size, polycarbonate membrane, 12 mm diameter, Corning Costar Corp.) were placed into each well of a prewarmed 12-well tissue culture plate (Corning Costar Corp.) that was filled with 1.5 ml of complete medium with or without OP-1. The suspension (≈1.5 ml) of alginate-recovered cells and their associated matrix (present from 75 beads) was seeded onto each insert (Fig. 1). Alternatively, freshly isolated chondrocytes (3×10^6 cells per insert, approximately the number of cells in 75 beads of alginate culture) were seeded directly onto an insert in the same manner as the alginate-recovered cells. After 14 additional days in culture (referred to as days 8–21 of culture), each insert was removed from the tissue culture plate and placed into a petri dish. The polycarbonate membrane was cut along its circumference using a scalpel, the

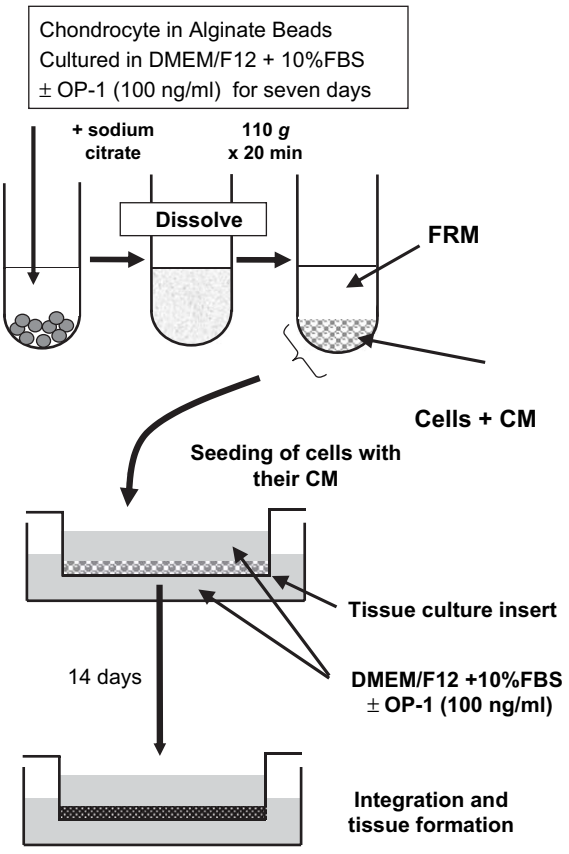

Fig. 1. ARC method. Chondrocytes were cultured in alginate beads with complete medium (DMEM/F12 containing 50 μg/ml gentamicin and 360 μg/ml L-glutamine) containing 10% FBS in the presence or absence of rhOP-1 (100 ng/ml) for 7 days and then the beads were dissolved by adding sodium citrate buffer to recover the chondrocytes with their CM. The cells with their CM were seeded onto the tissue culture insert and cultured for 14 days.

tissue and the membrane was carefully peeled away releasing the *de novo* tissue.

HISTOLOGICAL CHARACTERIZATION OF CARTILAGE TISSUE FORMED *IN VITRO*

The ARC tissue samples were fixed in 10% formalin with 10% cetylpyridinium chloride and embedded in paraffin. Eight micrometer sections were cut vertically, the paraffin removed, and tissue rehydrated using a sequential wash of xylene followed by decreasing percentages of ethanol diluted in water. Sections were then stained with 0.1% toluidine blue (Polysciences, Inc., Warrington, PA) at pH 7.0. Stained sections were visualized using light microscopy.

BIOCHEMICAL CHARACTERIZATION OF THE CM AND CARTILAGINOUS TISSUE FORMED *IN VITRO*

Both the chondrocyte-CM samples and the cartilaginous ARC tissues were digested with papain[22] overnight and analyzed biochemically. The DNA content was measured after digestion of the beads with papain using the bisbenzimidazole fluorescent dye method (Hoechst 33258: Polysciences, Inc.)[23]. The content of sulfated PG and hydroxyproline in the papain digest of each matrix compartment was determined using a modified dimethylmethylene blue (DMMB: Polysciences, Inc.) dye-binding method[22] and an isocratic reverse phase high-performance liquid chromatography (RP-HPLC) after derivatization with phenylisothiocyanate (Pierce Chemical, Rockford, IL)[22], respectively. The collagen content in each sample was then estimated by multiplying the hydroxyproline content by 8.2[24]. The relative ratio of PG to collagen was also calculated. To characterize collagen types synthesized and accumulated by the cartilaginous ARC tissues, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and fluorography after radiolabeling were performed at the end of culture as described previously[10].

STATISTICAL ANALYSES

All values are reported as the mean ± standard error of the results of analysis. To assess the effects of the inclusion of OP-1 and duration of culture (where appropriate) on biochemical constituents, the combined data of the separate experiments (three for alginate beads and five for ARC tissue) were analyzed by analysis of variance (ANOVA) with experiment as a random variable. When significant differences were detected, hypothesis testing (unpaired *T*-test) was performed to compare values at each day for the two culture conditions.

Results

FORMATION OF THE CM IN ALGINATE BEADS

During the 14 days of alginate bead culture, the cell number and composition of the CM changed significantly in both OP-1-treated and untreated beads (Fig. 2). The DNA content of the control beads increased 2-fold, while that of OP-1-treated beads increased 2.5-fold, which was significantly higher compared to the control beads ($P < 0.01$) [Fig. 2(A)]. In OP-1 treated beads, the magnitude of increase in PG content was significantly higher than that of the collagen content (PG: 212% of control; collagen: 121% of control [Fig. 2(B) and (C)]). When the results were expressed per DNA, essentially the same results were obtained for the PG content [Fig. 2(E)]. However, the collagen content did not increase when expressed per DNA [Fig. 2(F)]. After 7 days, the PG/collagen ratio was significantly higher in the OP-1 culture than in the control culture [Fig. 2(D)]. The 7-day culture period was selected for further studies with the goal to obtain enough matrix to produce ARC tissues with a minimum culture period. A culture period longer than 7 days resulted in the proliferation of cells, some of which escaped from the alginate beads and adhered to the culture dish as a monolayer. An apparent difference with OP-1 treatment was the increased size of the pellet resulting after isolation by a mild centrifugation. Cell viability, assessed using the LIVE/DEAD® cell assay, was not affected by the release of the chondrocytes from the alginate beads with dissolving buffer, or by inclusion of OP-1 in the culture medium (both groups, >95% cell viability). Histological analysis with alcian blue staining revealed that the structure of the CM around the chondrocytes was preserved well after the release from the alginate beads in both the OP-1 and control groups and that the CM around chondrocytes cultured in the presence of OP-1 was more voluminous (data not shown).

GROSS APPEARANCE OF THE ARC TISSUE FORMED *IN VITRO*

After 7 days of culture in alginate beads, the cells with their CM were released from the alginate and seeded

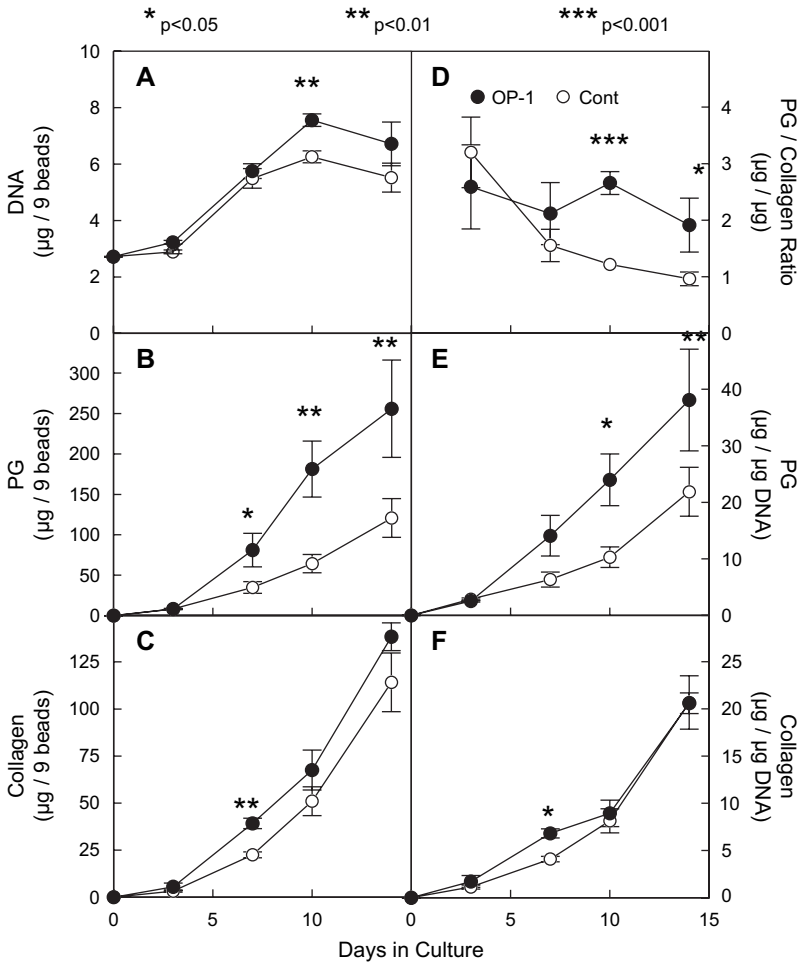

Fig. 2. Changes in DNA content and matrix accumulation in the CM of chondrocytes in alginate bead culture over time. Bovine articular chondrocytes were cultured in alginate beads in complete medium with or without rhOP-1 suplementation (100 ng/ml) for up to 14 days. At each time point (3, 7, 10 and 14 days), the DNA (A), PG (B) and collagen (C) contents of the CM were measured by the Hoechst dye assay, the DMMB method and RP-HPLC, respectively, as described in the Material and methods section. The PG/collagen ratio was calculated (D). The data of PG (E) and collagen (F) content were also expressed as µg DNA. The data points represent the mean ± standard error.

onto a culture insert. Over an additional 14 days of culture, the cells with their CM became progressively integrated into a single mass of cartilaginous tissue, or ARC tissue, by day 14. The tissue formed from ARCs cultured in the presence of 10% FBS alone was thin and fragile and could not be removed from the culture insert without damaging it. The tissue formed when cells were directly cultured on an insert, without preculture in alginate, was also thin and fragile and could not be removed from the insert even when cultured in the presence of OP-1. On the other hand, chondrocytes that were precultured in alginate and cultured on an insert in the presence of 10% FBS plus OP-1 produced a thick tissue that was white, opaque, and easy to handle and remove from the insert.

HISTOLOGICAL CHARACTERIZATION OF THE *IN VITRO* FORMED CARTILAGE CONSTRUCTS

The tissue formed by alginate-recovered cells cultured in the presence of OP-1 [Fig. 3(B)] was thicker than tissue formed by alginate-recovered cells cultured without OP-1 [Fig. 3(A)]. Histological examination of the thicker tissue revealed that it contained a cartilage-like matrix that stained strongly with toluidine blue, indicating the presence of PG molecules [Fig. 3(B)]. The tissue formed from alginate-recovered cells in the absence of OP-1 had a looser, less abundant matrix and a higher cell density [Fig. 3(A)]. The chondrocytes within all tissues produced from alginate-recovered cells retained a spherical shape, except for a thin layer of flattened cells at both surfaces [Fig. 3(B)]. The tissue appeared well integrated and the matrix had a homogenous appearance, with no distinctive demarcation (e.g., between the originally seeded CM regions). When cells were directly cultured on the insert in the presence of OP-1 for the same culture period, a thin tissue with little matrix was formed, even in the presence of OP-1 [Fig. 3(C)].

BIOCHEMICAL CHARACTERIZATION OF THE *IN VITRO* FORMED CARTILAGE CONSTRUCTS

On day 14 after transfer to the culture insert, alginate-recovered cells cultured in the presence of OP-1 yielded a tissue with a greater mass than that of alginate-recovered cells cultured in the absence of OP-1. The dry weight of the ARC constructs was significantly higher in the OP-1 treated cultures than that in the control culture (194% of control,

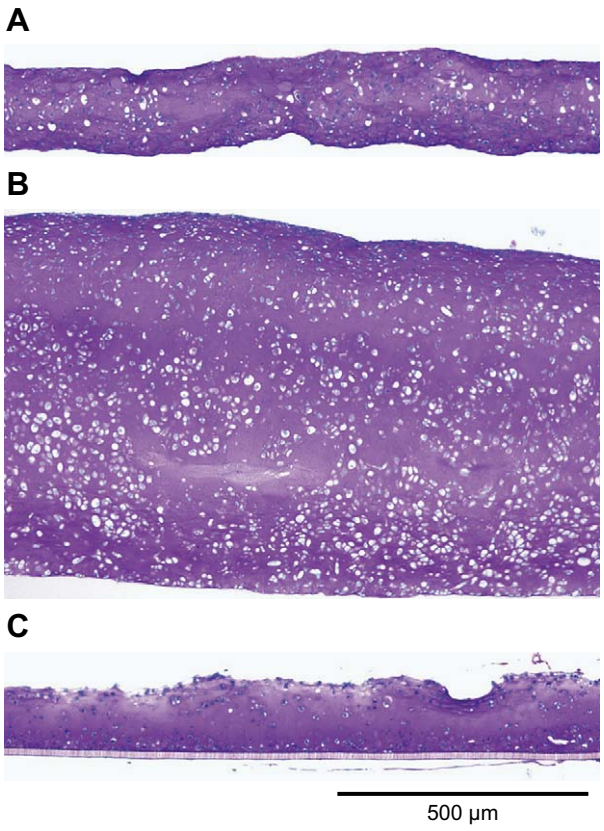

Fig. 3. Histological appearance of ARC tissue after 14 days of culture. The chondrocytes were precultured for 7 days in alginate beads and then transferred to a tissue culture insert as described in the Material and methods section. Cultures were fed complete medium (A) or complete medium supplemented with OP-1 (100 ng/ml) (B). The same culture medium was used during the alginate bead and insert cultures. The tissue formed was histologically assessed by staining with toluidine blue. The tissues formed with complete medium alone (A) or with cells alone (no alginate preculture) were seeded onto an insert (C), were processed while stil on the membrane due to their thin, fragile state.

$P < 0.001$) [Fig. 4(A)]. The water content of the OP-1 treated construct was significantly higher than that of the control construct (control, 92.4%; OP-1, 95.9%; $P < 0.001$ [Fig. 4(B)]). The PG [Fig. 4(C)] and collagen [Fig. 4(E)] contents expressed as per construct were also significantly higher in the OP-1 treated cultures (PG: 334% of control, $P < 0.0001$; collagen: 217% of control, $P < 0.001$). When the results were expressed per dry weight, only PG contents were significantly higher in the OP-1 treated culture than in the control culture (PG: 174% of control, $P < 0.0001$ [Fig. 4(D)]; collagen: 112% of control, n.s. [Fig. 4(F)]). The ratio of PG to collagen was higher in the OP-1 treated construct ([Fig. 4(G)], 172% of control, $P < 0.01$).

COLLAGEN TYPE ANALYSIS

To confirm the preservation of the chondrocytic phenotype, the construct was radiolabeled with ^{3}H-proline and the typing of newly synthesized collagen in the tissue and in the labeling media by SDS-PAGE fluorography was performed. Both fractions contained a similar prominent band at 95 kDa, corresponding to $\alpha 1$ chains (data not shown) as shown previously[10].

Discussion

We have shown here the effects of including the growth factor, OP-1, when using the ARC method to form a cartilaginous tissue. The results of these studies demonstrate that OP-1 can significantly enhance the capacity of adult chondrocytes to reform a cartilaginous tissue. The content of matrix molecules accumulated by chondrocytes during alginate bead culture was significantly enhanced by the inclusion of OP-1. Further, the OP-1-stimulated cells, recovered from alginate culture with an abundant CM, produced a more voluminous cartilaginous tissue compared to tissue produced by cells not stimulated with OP-1. The thickness of the tissue produced from the alginate-recovered cells cultured in the presence of OP-1 was approximately four times that of tissue cultured in the presence of 10% FBS alone.

While there is evidence that fetal articular chondrocytes maintained in high-density monolayer cultures are capable of reforming a cartilage-like tissue within a few weeks, their adult counterparts are much less effective in doing so[8,9]. In order to test the feasibility of stimulating the formation of a cartilaginous tissue by a growth factor, primary adult bovine chondrocytes were selected as the cell source in this study because the biological behavior of these cells in alginate was well characterized[17,19] and the culture period after seeding on the tissue insert was limited to 14 days. This study, using five different cell preparations, clearly shows the consistent capability of OP-1 to enhance the formation of a cartilaginous tissue that is suitable for cartilage transplantation over a short time period. The addition of OP-1 may also help overcome some of the differences in the biological response of chondrocytes among donors, in particular, differences related to age and the metabolic activity of the donor chondrocytes. However, because the metabolic activity of human chondrocytes is lower than that of chondrocytes of animal origin, it remains to be determined whether human chondrocytes can form a similar transplantable cartilage that can be handled surgically. The study by Nishida *et al.*[18], which qualitatively demonstrated that OP-1 stimulates the formation of a CM by human chondrocytes, suggests that a transplantable cartilaginous tissue is obtainable under certain culture conditions. Further studies of an effective duration of culture and dosage of OP-1 both in alginate beads and in the formation of the tissue on the semipermeable membrane using human chondrocytes with a range of ages are required for the future application to chondrocyte transplantation in human.

Because of its high cellular density and low collagen content, cartilaginous ARC tissue stimulated with OP-1 more closely resembles a fetal or immature articular cartilage than a mature articular cartilage[25]. At all culture durations in alginate, with or without OP-1 in the medium, the ratio of PG to collagen in the CM was higher [Fig. 2(D)] than that found in normal bovine articular cartilage ($\approx 0.20-0.25$)[26]. Similarly, in both OP-1 treated and control groups, the ratio of PG to collagen [Fig. 4(G)] was much higher in constructs than that found in normal bovine articular cartilage[26]. In addition, although the cells were from mature animals, chondrocytes were dividing in the presence of OP-1. Because immature cartilage has a high capacity for intrinsic repair when injured[27], those shared characteristics of cartilaginous ARC tissue in matrix composition and immature cartilage may promote the healing process, such as integration into the host cartilage and a proliferative response, after implantation. Furthermore, Petit *et al.*[24] have shown that the CM lacks a high concentration of collagen crosslink during short-term culture, which

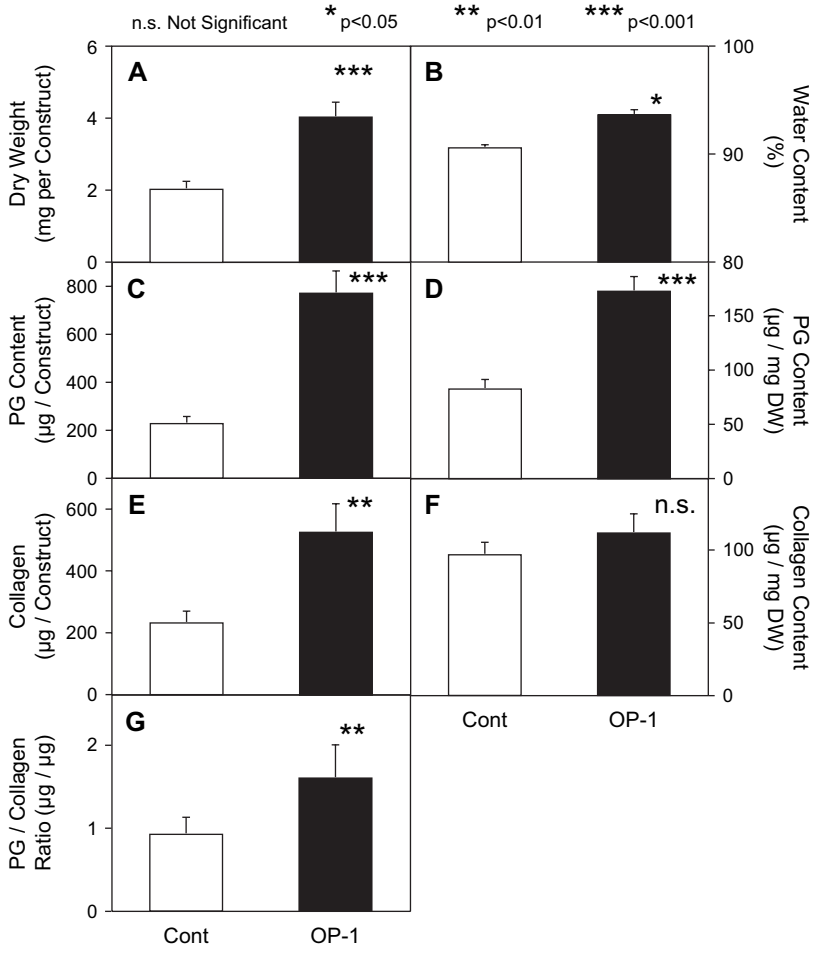

Fig. 4. Biochemical characteristics of the ARC tissue after 14 days of culture. After 14 days in culture on the insert (12 mm diameter), the ARC tissues were removed from the insert and separated from the membrane. The dry weights (A) and wet weights of the ARC tissues were measured and water content calculated. After papain digestion, the contents of PGs and collagen of the ARC samples were measured as described in the Material and methods section. The data of PG (D) and collagen (F) content were also expressed per dry weight. The dry weight (A), water content (B), PG (C) and collagen (E) contents per construct, PG content (D) per dry weight and PG/collagen ratio (G) were significantly higher in the ARC tissue cultured in the presence of OP-1. The data points represent the mean ± standard error.

may be beneficial for integration. However, it remains to be answered by *in vivo* studies whether mature chondrocytes can repair as effectively as fetal articular chondrocytes[27]. Because cellular density, matrix composition and mechanical properties of the cartilage constructs are dependent on the duration of culture and culture conditions, further *in vitro* and *in vivo* studies are necessary to test this hypothesis.

In autograft procedure a small surgical sample is procured from a non-weight bearing region of the joint[28]. The size of such samples necessitates cell expansion prior to tissue production. Since monolayer-expanded chondrocytes dedifferentiate, as indicated by the increased production of type I collagen[3], the effect of OP-1 on dedifferentiated chondrocytes may be different. The present study does not address the effect of OP-1 on the formation of cartilaginous tissue using monolayer-expanded chondrocytes. The addition of growth factors, such as OP-1, may benefit the redifferentiation process and subsequent production of cartilaginous tissue. The effects of OP-1 on the redifferentiation process and matrix formation of monolayer-expanded cells should be elucidated in detail to optimize the production of cartilaginous tissue for autograft application.

The focus of this study was on the biochemical and histological properties of the cartilaginous tissues formed *de novo*. To obtain assurance of clinical applicability, a more detailed assessment of the biomechanical properties of the ARC constructs is necessary. In addition, to further decrease the overall culture time and to increase the quality of the *in vitro* engineered tissue, other mechanisms that promote matrix formation during both steps of the ARC method, such as stimulation by other growth factors or a combination of growth factors, as well as different physical stimuli[29–31] may be useful.

Many growth factors, such as insulin-like growth factor-I (IGF-I), TGF-β, fibroblast growth factor (FGF), platelet-derived-growth factor (PDGF), and BMPs have been shown to enhance matrix formation and production and cell proliferation[32]. Certain combinations of growth factors, not limited to OP-1, during *in vitro* tissue formation may enhance the formation of a transplantable cartilage that is functionally mature and able to integrate with surrounding tissues *in vivo*. Finally, for the clinical application of tissue-engineered cartilage, the reproducibility of production of human cartilaginous tissue and the feasibility of minimizing the length of the culture period by using growth factors need to be addressed.

Acknowledgments

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